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13. ABSTRACT *(Maximum 200 words)*

Our team has pursued preclinical and clinical investigations combining the administration of IL-2 with tumor reactive monoclonal antibodies. Preclinical models, developed by our collaborators R.Reisfeld and S.Gillies, have shown the antitumor efficacy of antibody-IL2 fusion proteins in murine models. The KS-IL2 fusion protein recognizes the KSA antigen expressed on a broad range of human carcinomas, including breast cancer and is effective at preventing outgrowth of the KSA positive, syngeneic CT26-KSA carcinoma cell line in mice. The experiments now underway in this research study are designed to help improve the future clinical efficacy of the KS-IL2 reagent by clarifying its actions, testing its efficacy in murine models simulating pitfalls with human immunotherapy, and translating these results to human application through *in vitro* studies. These goals will be achieved through the following specific aims: 1) Clarify the distinct roles for NK cells and T cells in fusion protein induced antitumor efficacy in vivo and the dependence of this interaction on MHC expression; 2) Determine the molecular and cellular action of the KS-IL2 fusion protein *in vivo* and *in vitro*; 3) Assess KS-IL2 efficacy in murine models that simulate the pitfalls of clinical immunotherapy; 4) translate the data from mouse models to clinical application, through in vitro studies with human lymphocytes and breast cancer cells.

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Jack Gan 10/29/98

PI - Signature Date

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INTRODUCTION :

New directions in alternative treatment of breast cancer involve the use of monoclonal antibodies directed against breast tumor associated antigens. Several types of these antigens have been recently identified. These include the epidermal growth factor related HER-2/neu proto-oncogene and cell surface antigens such MH-99 glycoprotein and TAG-72 from the mucin family. These molecules have been examined as a targets for disease detection, radioimaging and immunotherapy. Numerous studies have demonstrated the value of antibodies against these antigens in prognostic and diagnostic applications, and have shown potential utility in animal models, *in vitro* settings, and in early clinical experiments. Using recombinant DNA technology, it has been possible to extend the utility of these molecules by altering their configuration to better suit specific needs. (1)

Extensive investigation is taking place specifically in the area of the use of antibodies to serve as delivery vehicles for immune stimulating agents that can target tumor cells for destruction directly or by attracting and stimulating resident immune effector cells. We have been interested in examining the immunotherapeutic effectiveness of antibody-cytokine fusion proteins. In these molecules the antibody portion recognizes the tumor associated antigen, and is covalently linked to a potent immune stimulator. (2) Initially we were studying the CC49-IL2 fusion protein, which had been difficult to use because of difficulty in obtaining sufficient quantities of protein for *in vivo* study. Thus we pursued *in vivo* delivery of this fusion protein by gene transfer. Unfortunately, as summarized in last year progress report, the level of gene expression (i.e. local and systemic CC49-IL2 protein levels) was insufficient for *in vivo* anti-tumor efficacy. Furthermore, a separate fusion protein (KS-IL2, see below), which is highly reactive with breast cancer, is now available to us in sufficient quantities to pursue the pre-clinical *in vitro* and *in vivo* studies we initially proposed with CC49-IL2. Thus our progress report, and planned studies reflect the more rapid progress we are making with the KS-IL2 fusion protein.

The KS1/4 murine monoclonal antibody (mAb), generated initially by R.Reisfeld and colleagues is reactive with a membrane molecule found to be heavily expressed on human cancer cells including breast, lung, colon and prostate. (3) The MH-99 antigen recognized by this antibody is EP-CAM molecule, strongly present on adenocarcinoma cells, and with low level of expression on certain normal epithelial cell types including mucin rich cells of the GI system. (4) Preliminary clinical testing of the KS1/4 antibody in patients with measurable disease showed relatively little toxicity or antitumor effect. (5) We intended to test preclinical strategies utilizing a molecular derivative of the KS1/4 antibody, designed to provide enhanced clinical efficacy. Two major molecular modifications within the molecule were created: first - grafting the murine hypervariable regions (responsible for antigen binding) to a human IgG framework to achieve a "humanized" KS1/4 antibody able to mediate ADCC. Secondly, linking human interleukin-2 by genetic construction to the carboxy terminus of each heavy chain of this humanized antibody, completed the design of the fusion protein, designated huKS-IL2.(6) The principal action of this new molecule is the activation of the immune system through IL-2 and Fc receptors, localized to tumor cells by the fusion protein's antibody component. This fusion protein has been recently shown to be able to mediate potent antitumor effects against established lung and liver metastases

in syngeneic mice bearing tumors which express the KSA (MH-99) antigen.

This syngeneic tumor model created for evaluation of the humanized KS-IL2 fusion protein is based on the CT26 colon carcinoma, which originated in BALB/C mice. This is a weakly immunogenic tumor that can induce a protective immune response following appropriate syngeneic immunization. (7) The variant of these tumor cells , CT-26/KSA has been achieved after transfection of parental cells with the human KSA gene, and expresses the KSA molecule on the cell surface, allowing the recognition by the KS1/4 antibody. (8) Following intravenous or intrasplenic injection of these cells into animals, metastases arise in the liver as well as lungs and are rapidly fatal. Animals treated with the KS1/4 antibody together with IL-2 show a striking decrease in the number and size of their metastases; however, considerable metastatic disease remains. In contrast, daily intravenous injections of the hu KS-IL2 fusion protein for seven days causes complete elimination of all detectable lung and liver metastases. (6) Animals that are long term survivors following this fusion protein treatment can be rechallenged with the parental CT26-KSA tumor cells and show rapid tumor rejection *in vivo.*

As this tumor associated antigen is present on breast adenocarcinoma cells as well, we attempted to utilize the animal model described above to elucidate pharmacokinetics of huKS-IL2 fusion protein and assess the potential of induction of neutralizing antibodies against fusion protein in injected animals. Our ultimate goal is to determine how this molecule is likely to be most effective in treating clinical cancer and which immune mechanisms are likely involved in this process.

BODY :

The following is a summary of progress we have made over the past year in this experimental system.

1. ***Analysis of the ability of huKS-IL2 fusion protein to specifically recognize EP-CAM (+) tumor cells as well as ability to deliver the cytokine to their surface.***

Using flow cytometry technology the KS-IL2 fusion protein was evaluated. Indirect staining of the T47D breast tumor cell line and LS174 colon tumor cell line after incubation with fusion protein showed that the huKS-IL2 fusion protein binds to the tumor cells, bringing the IL-2 component of the molecule to the cell surface in a form recognized by anti-human IL-2 antibody. This experiment proved that the fusion protein can act as a delivery vehicle of cytokine to the tumor site. (FIG. 1)

2. ***Assessment of ability of huKS-IL2 fusion protein to stimulate proliferation of IL-2 dependent cells in vitro.***

For this purpose fusion protein was tested as a stimulus for the TF-1β cell line, created previously in the lab, as well as PHA stimulated PBMCs from a healthy donor. Activity of the fusion protein IL-2 component was compared to a standard preparation of recombinant human IL-2 and another fusion protein (hu14.18-IL2) of different tumor specificity (to the GD-2 antigen) in a 72 hour proliferation assay. The TF-1β cell line responds to IL-2 using intermediate affinity IL-2 receptor complexes. The other responding cell population, peripheral blood mononuclear cells (PBMC) were stimulated previously with PHA mitogen to generate expression of high affinity IL-2 receptors. Fusion protein huKS-IL2 induced comparable proliferation (to soluble IL-2 or hu14.18-IL2 fusion protein) of human cells expressing intermediate or high affinity IL-2 receptor complexes. This experiment proved that the IL-2 component of huKS-IL2 fusion protein retains its functional activity within the fusion protein molecule. (FIG. 2)

3. ***Analysis of the specificity in mediating tumor cell destruction by huKS-IL2 fusion protein.***

For this experiment freshly obtained PBMCs from healthy donors were used as effector cells in a 4 hour chromium release assay with T47D human breast carcinoma and LS174 human colon carcinoma targets. As control target cells the M21 human melanoma cell line and LAN-5 human neuroblastoma cell line served. The cytotoxicity mediated by serial dilutions of effector cells was measured on labeled targets in the presence of culture media, human recombinant IL-2 , huKS-IL2 fusion protein and hu14.18-IL2 fusion protein. The results from this experiment indicate that huKS-IL2 fusion protein specifically binds to the T47D and LS174 tumor targets and interacts with the effector cells to facilitate ADCC. Furthermore in this experiment, target cell destruction was superior in the presence of fusion protein in comparison to the IL-2. Also, the huKS-IL2 fusion protein does not bind to the M21 melanoma cells or to the LAN-5 neuroblastoma cells, thereby demonstrating specificity. (FIG. 3)

4. ***Analysis of ability of fusion protein to facilitate antibody-dependent tumor cell destruction (ADCC) by PBMCs obtained from patient after IL-2 therapy.***

Cryopreserved PBMCs obtained from patients subjected previously to IL-2 therapy were used in this experiment as effector cells to analyze ADCC in the presence of fusion protein. The breast cancer cell line T47D, was labeled with ^{51}Cr and used as a target for destruction. Results obtained showed that huKS-IL2 fusion protein induces striking ADCC, in comparison to the KS1/4 antibody used with soluble IL-2 as separate molecules present in the *in vitro* system. Separately, when the Mikβ-1 antibody (specific against the IL-2 receptor β subunit) was used in the assay, this ADCC was significantly inhibited. This result indicates that the IL-2 receptor complex is involved in the boosted killing of target cells, induced by the huKS-IL2 fusion protein. (FIG. 4)

5. ***Creating the assays for specific detection of the intact fusion protein and its components.***

For *in vitro* as well *in vivo* studies we established very sensitive and specific enzyme-linked immunosorbent assay (ELISA) systems that allow the accurate detection of intact fusion proteins, by requiring simultaneous recognition of the IL-2 and immunoglobulin components of the fusion protein molecule. (9) Detection methods based on recognition of the immunoglobulin component and on the recognition of the cytokine component of the fusion protein were also developed. All three of these systems are very dependable and useful in evaluating fusion protein *in vitro* in buffer, in tissue culture media or even in mouse serum, and can be applied also to *in vivo* experiments. The specificity of these assays allowed us to reliably determine nanograms per milliliter quantities of intact fusion proteins even in the presence of degradative products (which might occur *in vivo*) or free immunoglobulin or free cytokine. A summary of these assays is presented in **Table I**. The huKS-IL2 fusion protein can be easily detected by the IL-2/IgG1 ELISA (which detects the intact fusion protein molecule), by the IgG1 ELISA (which detects the immunoglobulin component of the fusion protein) and by the IL-2 ELISA (which detects the cytokine component of the fusion protein).

6. ***Evaluation of neutralizing anti-FP antibodies in injected animals.***

It was anticipated that a strong mouse anti-humanized antibody response will arise in conventional mice treated with huKS-IL2 fusion protein. To evaluate this, a group of mice was analyzed during the treatment (15 μg/day intravenous injections of KS-IL2 for 5 consecutive days). Blood samples were collected at day 0, 2, 4 and 7 of treatment. Serum was evaluated for the presence of mouse anti-human antibodies (MAHA) by ELISA method using plates coated with human IgG1. There was a rapid elevation of anti-human IgG antibodies in mouse serum after 4 days of treatment with huKS-IL2 fusion protein. (FIG. 5)

These data would be relevant to *in vivo* protocols designed to provide enhanced antitumor efficacy through the prolonged use or repeated cycles of treatment proposed for the huKS-IL2 molecule, if this MAHA response was found to influence the levels of KS-IL2 in previously treated mice.

7. ***Detection of fusion protein levels in mouse serum in naive and previously treated mice.***

Because of the potential clinical importance of the KS-IL2 fusion protein, pharmacokinetic studies were conducted using BALB/C mice. Naive animals were injected intravenously with 15 μg of fusion protein and blood samples from the tail vein were collected at time 0, 2, 8, 24, 48 and 144 hours after the i.v. bolus. The fusion protein was detected in mouse serum using the IgG1 ELISA (recognizing the immunoglobulin component of fusion protein). Peak level (2600 ng/ml) was demonstrated at 2 hrs after injection, and gradually diminished over time. (FIG. 6 - "control mice"). Additionally, a separate group of animals received prior treatment with huKS-IL2 fusion protein for 5 days at the dose of 15 μg /day (as shown in FIG. 5). Two weeks later these same animals received a single 15 μg bolus of fusion protein. Blood samples were collected at the same time points as for the naive animals and serum was evaluated in the IgG1 ELISA. Surprisingly, no detectable serum level of fusion protein in these samples was found, except for a very low concentration (42 ng/ml) found at 2 hrs after the infusion. (FIG. 6 - "experimental mice"). These very different clearance patterns between these two groups of animals indicated that the MAHA response shown in FIG. 5, was able to neutralize the KS-IL2 in previously treated animals.

8. ***Molecular and cellular testing of KSA antigen positive cells used for in vivo experiments in murine model of human cancer.***

Molecular analysis of the presence of the KSA transcript was performed on KSA-positive CT26-KSA tumor cells. After extraction of total RNA and reverse transcription, resulting cDNA was PCR amplified using primers specific for KSA and G3PDH. PCR products separated by agarose gel electrophoresis were evaluated and intensity of bands compared and corresponded copy numbers were calculated. In this system is possible to obtain a semi-quantitative detection of KSA transcripts, even if KSA (+) cells are found at a 1:100,000 ratio in the tested population. (FIG. 7) This powerful and extremely sensitive method will have future application in murine studies of undetectable disease such as the presence of micrometastases, difficult to evaluate by other techniques.

Cellular analysis of the CT26-KSA tumor cells was also performed using flow cytometry technology. Staining of these cells with humanized anti-KSA monoclonal antibody showed a strong signal on CT26-KSA cells (as well on a variant cell line designated as CT26-KSA21.6) as a result of the presence of the KSA molecule on the surface of these cells. Additionally, staining with monoclonal anti-H2Dd antibody (which recognizes mouse MHC class I antigen) was performed. The CT26-KSA21.6 variant showed much lower expression of MHC class I molecules in comparison to CT26-KSA cells. (FIG. 8) These two very well characterized cell lines are intended to use in further *in vivo* experiments to generate mouse model of human tumor and elucidate the mechanism of treatment with huKS-IL2 fusion protein.

CONCLUSIONS :

Over past year substantial progress has been made in our studies of antibody-IL2 fusion proteins potentially useful for treatment of human breast cancer. The huKS-IL2 fusion protein can specifically bind to human breast cancer cells and deliver IL-2 to their surface. It can activate IL-2 responsive cells, and induce tumor specific ADCC. We have adapted murine models that allow *in vivo* testing of KS-IL2 as a means for destroying KSA (+) tumor cells *in vivo*. Our next steps will be : 1) to evaluate the *in vivo* fate of the KS-IL2 molecule in mice, to determine whether the fusion protein remains intact or is degraded; 2) to evaluate the role of T cells and NK cells in mediating anti-tumor effects, and 3) to test the ability of pre-treatment with IL-2 or anti-CD3 antibody *in vivo* as effector cells activation as a means to augument antitumor efficacy seen with KS-IL2 fusion protein treatment.

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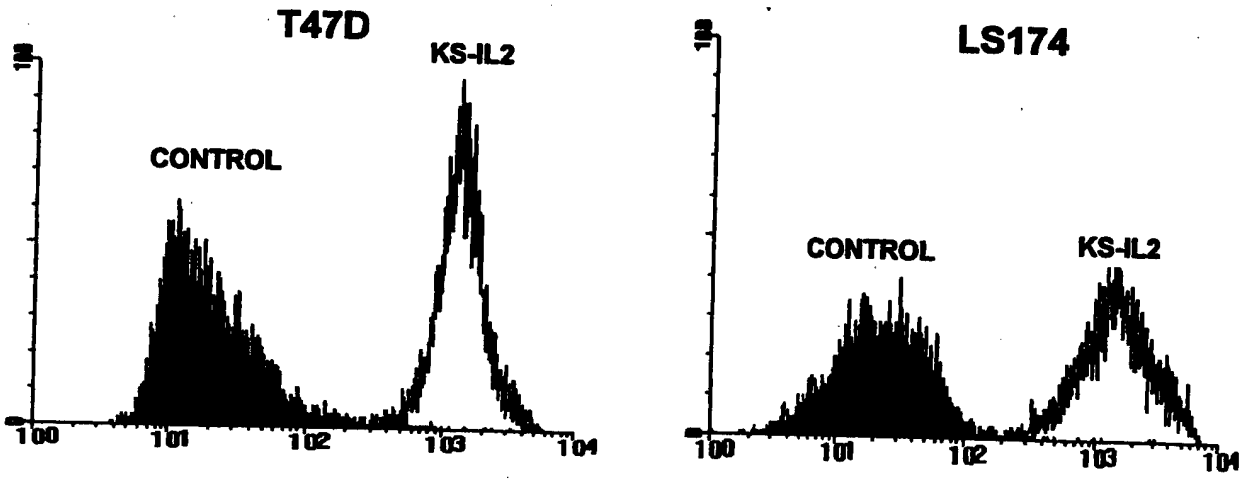
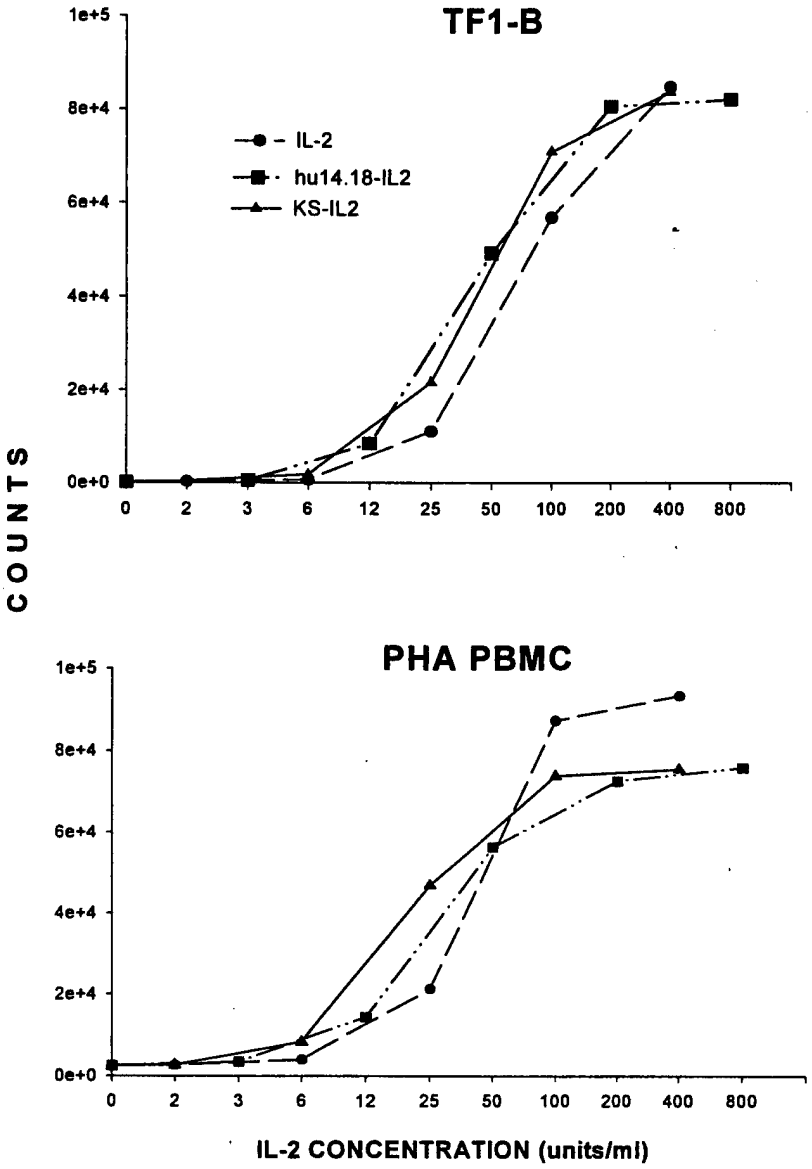
FIGURE 1 :

Delivery of IL-2 to Tumor Cells via Fusion Protein

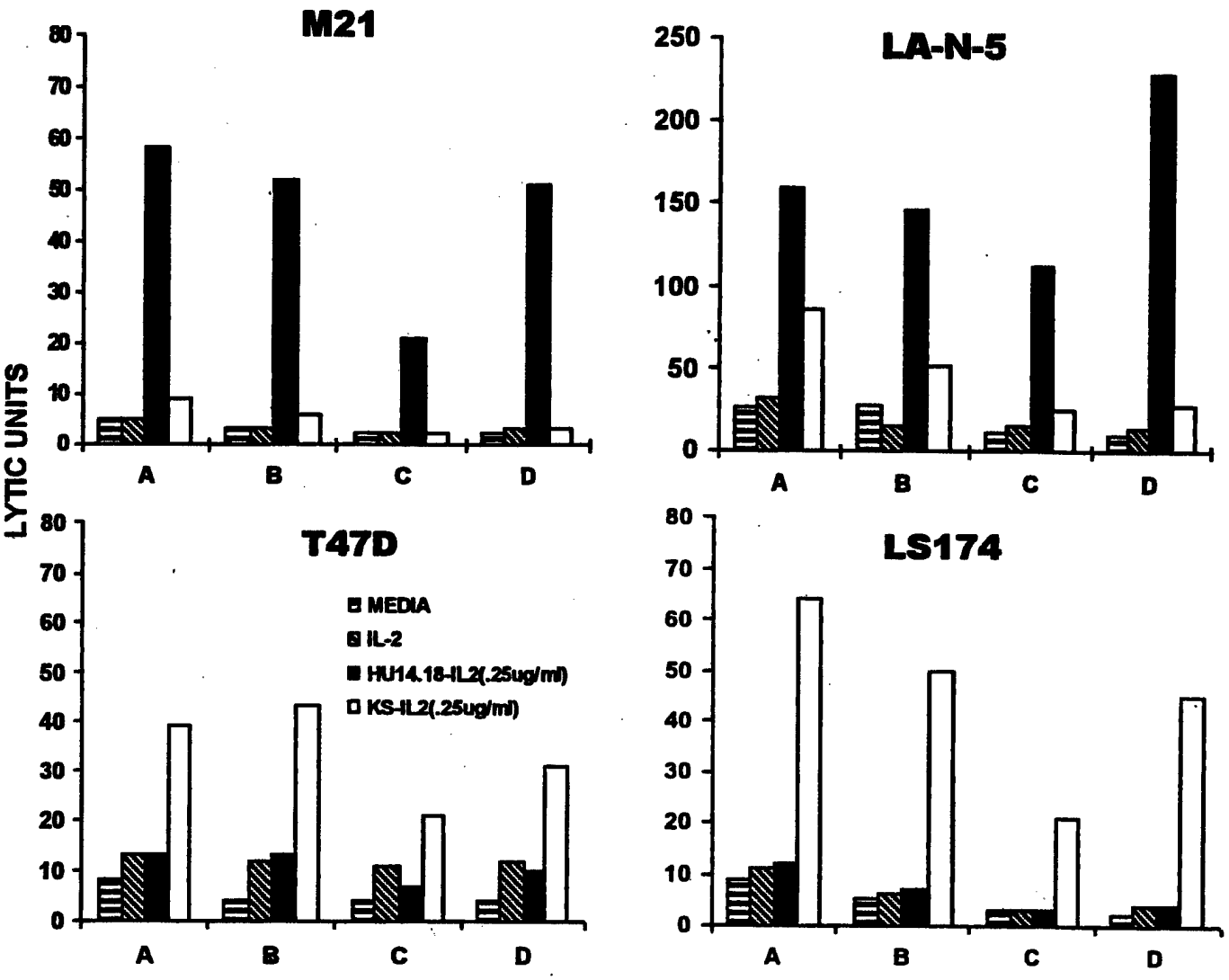


This figure evaluates the delivery of IL-2 to the surface of tumor cells by fusion protein. Indirect staining of the T47D breast and LS174 colon tumor cell lines shows that the KS-IL2 fusion protein binds to the cells and then the IL-2 end of the molecule is recognized by a rat anti-human IL-2 conjugated to phycoerythrin. The fusion protein was first incubated with the cells for 30 minutes at 4 degrees and then washed. A rat anti-human IL-2-PE was added for a 30 minute incubation and washed. The cells were then analyzed on a FACScan. These data show that the fusion proteins bind to the tumor cells bringing the IL-2 component to the cell surface in a form recognized by an anti-IL2 antibody.

Proliferation Induced by Humanized Fusion Proteins



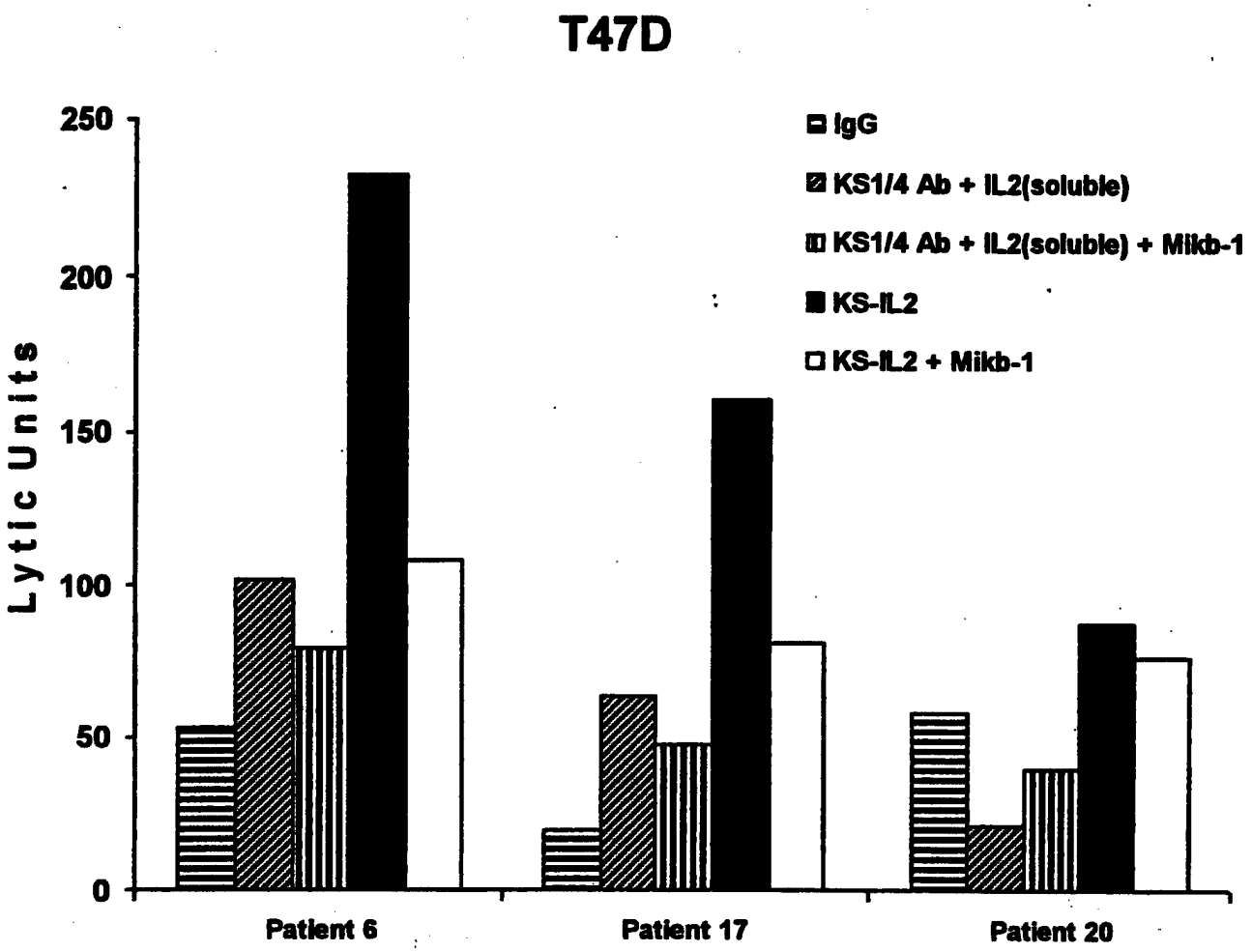
A 72 hour proliferation assay was used to compare the ability of the IL-2 within the KS-IL2 fusion protein to stimulate an IL-2 induced proliferative response. This response was compared to that of IL-2 alone and a second fusion protein hu14.18-IL2. One of the responding cell populations, Tf1-β, is a myeloid leukemic cell line transfected with the gene for the IL2 receptor β chain. This Tf1-β cell line responds to IL-2 using intermediate affinity receptor complexes. The other responding cell population is peripheral blood mononuclear cells (PBMC) previously stimulated with PHA to generate cells expressing high affinity IL-2 receptors. KS-IL2 induced comparable proliferation as soluble IL-2 or hu14.18-IL2 on human cells expressing intermediate or high affinity receptors.



Freshly obtained peripheral blood mononuclear cells from four healthy control donors (A,B,C,D) were used as effector cells in a 4 hour chromium release assay with M21 a human melanoma target, LA-N-5 a human neuroblastoma target, T47D a human breast carcinoma target and LS174 a human colon carcinoma target. The cytotoxicity mediated by serial dilutions of effector cells was measured on these target cells in media alone, in IL-2 (100U/ml), in KS-IL2 fusion protein (0.25μg/ml) and in hu14.18-IL2 (0.25μg/ml). The data, shown in lytic units, indicate that the KS-IL2 fusion protein specifically binds to the T47D and LS174 tumor targets and interacts with the effector cells to facilitate ADCC. Furthermore, the KS-IL2 fusion protein does not bind to the M21 melanoma or the LA-N-5 neuroblastoma cell line. The hu14.18-IL2 fusion protein binds to the M21 and LA-N-5 tumor target and results in good ADCC. The hu14.18-IL2 fusion protein does not bind to the T47D or the LS174 targets nor induce ADCC of them, thereby demonstrating specificity.

FIGURE 4 :

Tumor Bound Fusion Protein Facilitated ADCC Involves IL-2R Activation



Cryopreserved PBMCs from three patients obtained following *in vivo* IL-2 therapy were thawed and tested at varying dilutions as effector cells in a 4 hour ^{51}Cr release assay. The breast cancer target cell line, T47D, which expresses the KS antigen was first labeled with ^{51}Cr and washed one time. These ^{51}Cr treated target cells were then incubated for one hour at 4 degrees with human IgG(control), KS1/4 antibody, or the KS-IL2 fusion protein. The cells were washed twice before addition into the assay. Soluble IL-2 at 100 U/ml was added to the KS1/4 antibody coated targets for the 4 hour assay. For one group of assay wells of targets bearing KS1/4 plus IL-2 antibody or KS-IL2 fusion protein, 3 μg/ml of Mikβ-1 antibody (an antibody that binds the IL2Rβ chain and blocks IL-2 binding) was added. These results show that the KS-IL2 fusion protein induces striking ADCC, and that a component of this killing (especially for Patients 6 and 17) can be inhibited with the Mikβ-1 antibody. This indicates that the IL-2 receptor is involved in the boosted ADCC induced by KS-IL2.

FIGURE 5 :

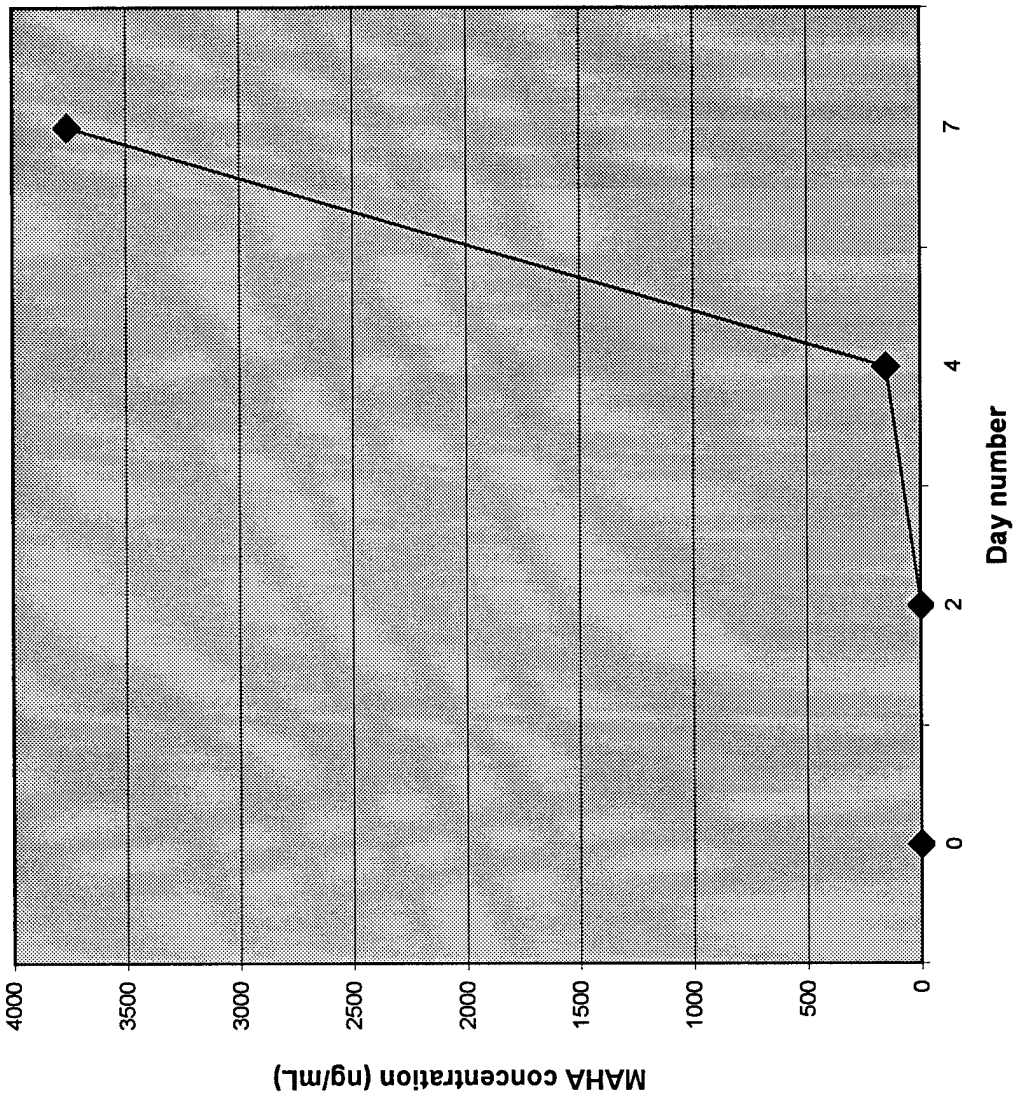
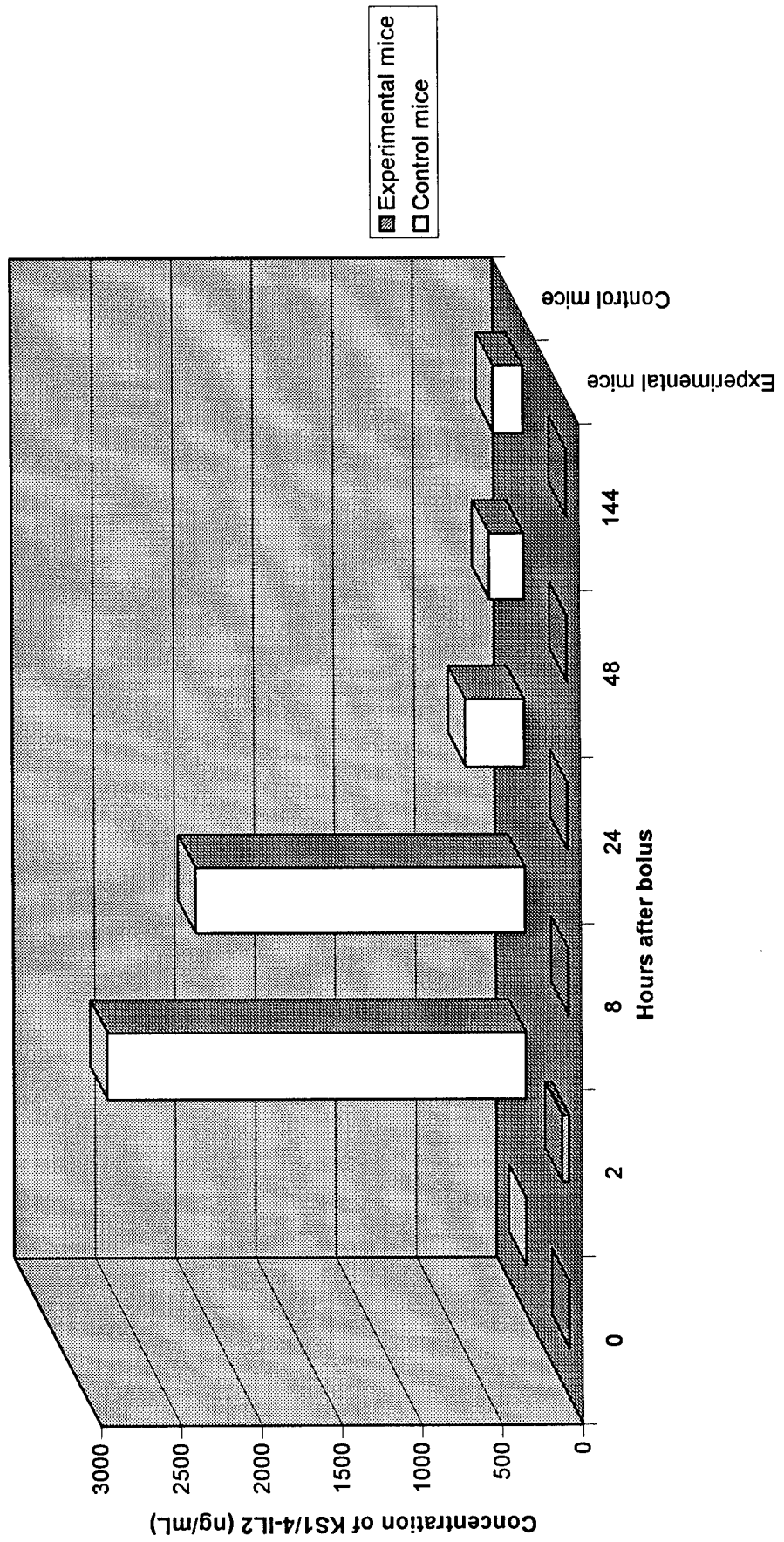
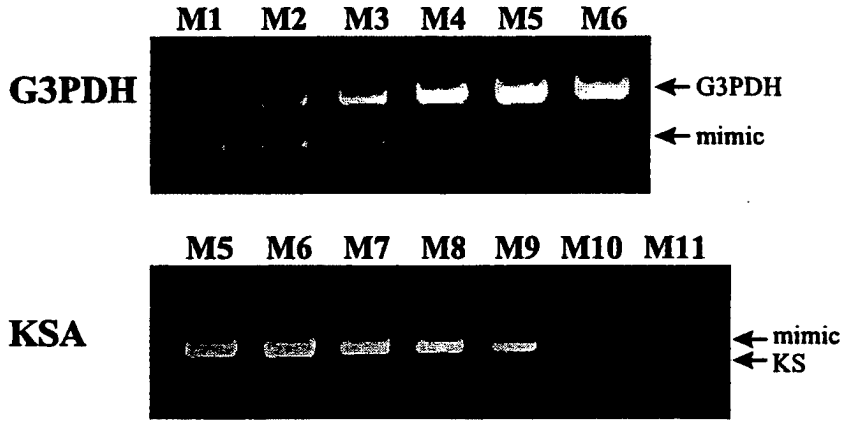

Mouse anti-human antibodies produced in response to KS1/4-IL2
MAHA concentration (ng/mL)
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
7
Day number

FIGURE 6 :


Concentration of KS1/4-IL2 in Mouse serum after 15 mcg bolus
Concentration of KS1/4-IL2 (ng/mL)
3000
2500
2000
1500
1000
500
0
Hours after bolus
0
2
8
24
48
144
Experimental mice
Control mice
Experimental mice
Control mice

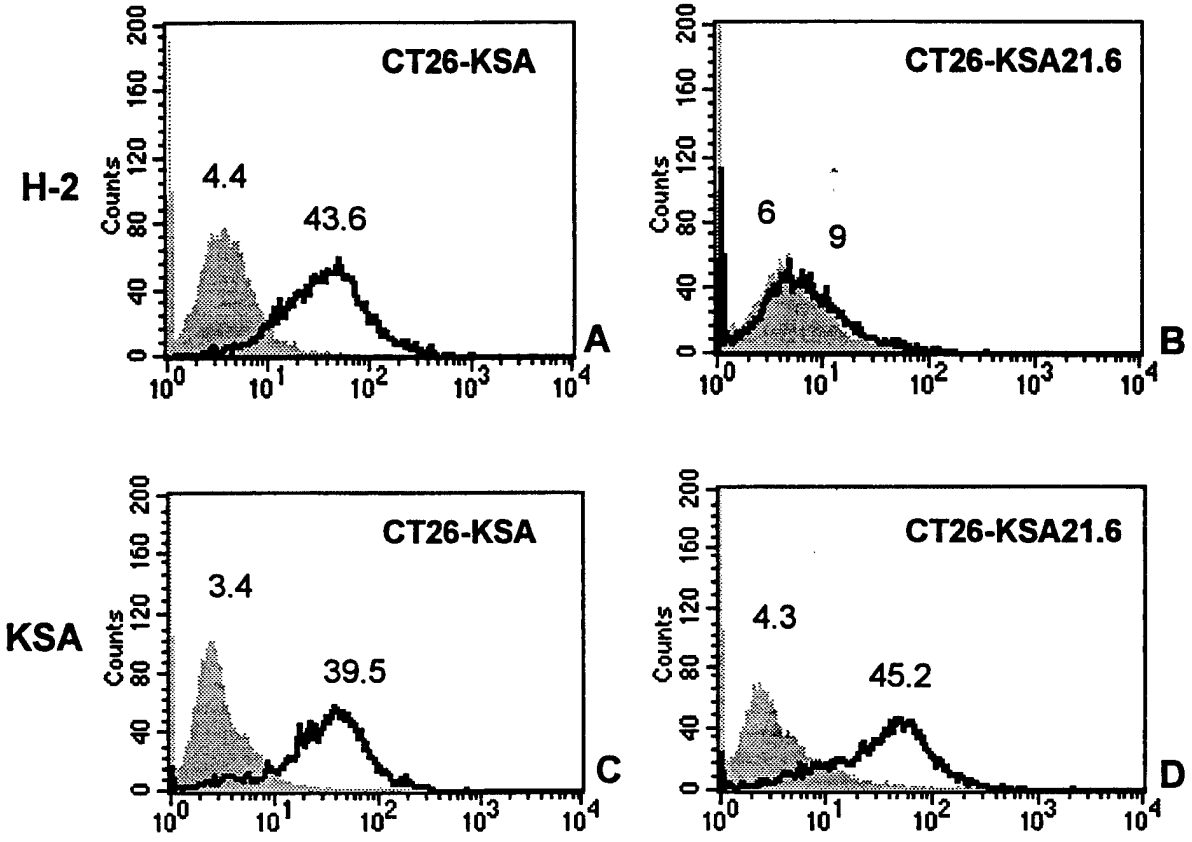
Quantitative PCR detection of CT26-KSA cells



Testing of QC-PCR sensitivity for the quantification of tumor cells in vivo. KSA-positive CT26-KSA tumor cells were mixed with KSA-negative 4T1 tumor cells at a ratio of $1:10^5$ followed by extraction of total RNA and reverse transcription. Resulting cDNA, mixed with serial dilutions of competitive fragment, was PCR-amplified using primers specific for G3PDH or KSA. PCR products as separated by agarose gel electrophoresis and ethidium bromide staining are shown. Comparison of band intensity was performed by eye. The upper panel shows the amplification of G3PDH which was used to normalize the amount of cDNA. The competitive fragment for G3PDH was used in 4-fold dilutions starting at M1=$3.01x10^6$ copies per reaction. Band equity was at dilution M2. The lower panel shows amplification of KSA and different 4-fold dilutions of the competitive fragment starting at M5=$1.94x10^4$ copies per reaction. Band equity was at dilution M11. This corresponds to 0.01 copies of KSA per 1000 copies of G3PDH. When multiplied with the dilution factor of 10^5 the number of KSA copies per 1000 copies of G3PDH is approximately 1000. The copy number of KSA per 1000 copies of G3PDH determined in total RNA from undiluted CT26-KSA cells (not shown) was found to be in the same range (700 KSA/1000 G3PDH). This system therefore shows that quantitative detection of KSA transcript is possible, even when contained only in cells found at a $1:10^5$ ratio in the tested population.

FIGURE 8 :

Expression of H-2 and KSA Molecules on CT26-KSA and CT26-KSA21.6 Detected by Flow Cytometry



MHC class I and KSA expression on CT26-KSA and CT26-KSA21.6 tumor cell lines determined by flow cytometry. A, staining of CT26-KSA cells with fluorescein isothiocyanate (FITC) labeled monoclonal anti-H2Dd antibody (solid line) and FITC-labeled mouse IgG2A isotype control antibody (gray shade). B, staining of CT26-KSA21.6 cells with FITC-labeled anti- H2Dd antibody (solid line) and FITC-labeled mouse IgG2A isotype control antibody (gray shade). C, staining of CT26-KSA cells with monoclonal humanized anti-KSA monoclonal antibody (primary staining) followed by FITC-labeled goat anti-human antibody (solid line) and secondary antibody alone (gray shade). D, staining of CT26-KSA21.6 with monoclonal humanized anti-KSA followed by FITC-labeled goat anti-human antibody (solid line) and FITC-labeled goat anti-human antibody alone (gray shade). All antibodies were used at a concentration of 1μg/10^6 cells. 10^4 live cells were analyzed using propidium iodide staining. Numbers shown inside graphs are mean fluorescence intensity values. In this, and all other flow cytometry figures, the x-axis is flourescence intensity, and the y-axis is cell number.

Table I : Specificity of fusion protein detection

ELISA Detection System	Protein Analyzed						
	CH14.18	CH14.18-IL2	CC49	CC49-IL2	IL-2	KS-IL2	hu14.18-IL2
"1A7/IL-2"	-	+	-	-	-	-	+
"IL-2 /IgG1"	-	+	-	+	-	+	+
"IgG1"	+	+	+	+	-	+	+
"IL-2"	-	+	-	+	+	+	+
"A.I. 49-3/ IL-2"	-	-	-	+	-	-	-

Specificity of the "1A7/IL-2" ELISA, the "IL-2 /IgG1" ELISA, the "IgG1" ELISA, the "IL-2" ELISA and the "A.I.49-3/IL-2" ELISA. Purified immunoglobulins (ch 14.18, or CC49), purified IL-2, or immunoglobulin/cytokine fusion proteins (ch14.18-IL2, CC49-IL2, KS-IL2, or hu14.18-IL2) were compared. Samples designated by (+) indicate protein was detected, at the expected level, in that assay system. Samples designated as (-) were below detection limits. Each result represents 2 to 3 replicate experiments.